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DEVELOPMENT OF NOVEL, REVERSIBLE, NON-TOXIC ANTICOAGULANTS FOR GREATLY EXTENDED PLATELET STORAGE

ANNUAL AND FINAL REPORT

Arthur P. Bode

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The project to develop a novel anticoagulant for preservation of blood platelets was carried through the in vitro testing stages before termination due to Congressional budget restrictions. The next phase would have begun the toxicity and efficacy studies in animals necessary for application of our findings to practical use in blood banking. We were able to demonstrate, in vitro, preservation of platelet function and integrity for 15 days at 22°C through the addition of protease inhibitors and inhibitors of platelet activation to the citrate anticoagulant. Many formulations were devised and tested; the best was composed of the additions of PGE-1 and theophylline to increase cyclic AMP plus a thrombin inhibitor (Thromstop) and a plasmin-kallikrein inhibitor (aprotinin). A lowering of the surface area-to-PC volume ratio was necessary to achieve full benefit of the inhibitors. Our results demonstrate that storage of platelets can in principle be remarkably improved by developing means to inhibit platelet activation. Keywords-

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SUMMARY

The overall goal of this project was the development and testing of an anticoagulant formulation that would permit storage of platelet concentrates for 15-20 days at 22°C. The first two years were focused on examining the effects on platelet function of a long list of inhibitors directed against the clotting enzymes thrombin and Factor Xa or compounds acting through cyclic AMP or other biochemical pathways to limit the effects of platelet activation stimuli. We found that the best anticoagulant was citrate supplemented with the combination of a thrombin inhibitor (like hirudin or Thromstop) plus aprotinin and the platelet activation inhibitors Prostaglandin E-1 and theophylline. This formulation was used to prepare platelet concentrates in which in vitro platelet function was well maintained for 15 days. We also found that a similar approach with these inhibitors in artificial storage media (esp. that of Holme et al., Br. J. Haematology 66:233-238, 1987), produced platelets exhibiting evidence in vitro of continued function and integrity over a 20 day storage period. This is now a standard against which other products are compared in our laboratory.

The next phase of the project would have included studies of acute toxicity of the anticoagulant formulation and in vivo testing of platelets for efficacy and circulation capacity (radiolabelled survivals and recoveries) after long term storage. Unfortunately, the termination of the contract July 30, 1988, by reason of Congressional budget limitations prevented the initiation of this part of the workplan. The questions still remain concerning the ability of platelets stored for 15-20 days in the novel anticoagulant to circulate properly and function in vivo. The data gathered from in vitro studies seems promising, but the direct testing must be done to prove the utility and safety of this product for the medical community.

Our results to date support the working hypothesis that platelet activation plays a major role in the loss of platelet function and integrity during storage, leading to the "storage lesion". The use of inhibitors to limit the occurrence and effects of platelet activation appears to slow down onset of the storage lesion, at least according to in vitro markers. The targeted useful shelf-life of 15 days for a platelet concentrate for transfusion purposes in the military medical setting may well be achievable with this strategy. It is regretable that the project was halted before progress could be made towards implementation of this approach for blood banking practice.

The data generated during the funded part of this project have been presented in several manuscripts and abstracts.

Publications

In press: (1) "Plasmin Activity and Complement Activation During Storage of Citrated Platelet Concentrates", J. Lab. Clin. Med.

(2) "Changes in the Activation State of Stored Platelets", chapter in Plasma Therapy and Transfusion Technology.

In review: (3) "Automation of Analysis of the Hypotonic Shock Recovery Rate in Platelet Suspensions", Vox Sanguinis.

(4) "Metabolic Status of Platelet Concentrates During Extended Storage: Improvement with Pharmacological Inhibitors and Reduced Surface-to-Volume Ratio", Vox Sanguinis.

(5) "The Effect of Protease and Platelet Activation Inhibitors on the Loss of Surface Glycoprotein Ib During Storage of Platelet Concentrates", Blood.

(6) "The Use of Thrombin and Factor Xa Inhibitors in the Preservation of Platelets Stored for Transfusion", J. Lab. Clin. Med.

In preparation:

(7) "Extended Storage of Platelets in Artificial Media with Platelet Activation Inhibitors".

(8) "The Effect of Container Surface-to-Volume Ratio on Extended Storage of Modified Platelet Concentrates".

Presentations (oral)

(1) "Conversion of Complement Factor C3 During Storage of Citrated Platelet Concentrates", N.C. Assoc. Blood Banks; Sept., 1986.

(2) "Generation of Complement Activation Peptides During Storage of Platelet Concentrates (PC)", International Congress on Thrombosis and Hemostasis; July, 1987.

(3) "Plasmin's Role in the Platelet Storage Lesion", Amer. Assoc. Blood Banks; Nov., 1987.

(4) "Effects of Plasmin and Thrombin Inhibitors and Surface-to-Volume Ratio on the Platelet Storage Lesion", Amer. Assoc. Blood Banks; Nov., 1987.

(Future)

(5) "The Loss of GPIb from Stored Platelets is Caused by Platelet Activation, Not Plasma Enzyme Activity", accepted for presentation at Amer. Assoc. Blood Banks; Oct., 1988.

(6) "Storage of Platelet Concentrates (PC) 20 Days with Platelet Activation Inhibitors in Artifical Media", accepted for presentation at Amer. Assoc. Blood Banks; Oct., 1988.

INTRODUCTION

Platelet concentrates (PC) have been used for several decades in civilian blood banks for treatment of patients having thrombocytopenia or dysfunctional platelets. For care of the combat casualty, platelet infusions would be indicated in cases of severe trauma with massive transfusions causing "washout thrombocytopenia", and in casualties with oblated bone marrow such as in severe radiation injuries. The availability of PC to the military surgical and medical theater has been greatly limited by the short shelf-life of only 5 days. From our previous work on the role of platelet activation in the "storage lesion" of platelet concentrates (Contract DAMD17-84-C-4005), it appeared possible to greatly extend the shelf-life of platelets by developing a means to prevent platelet activation or limit its effects during blood processing and storage. This project was thus centered on the evaluation of pharmacological and mechanical approaches to inhibit thrombin activity and its generation in PC as well as on general strategies to identify and block other agonists present in the system.

The first objective was to demonstrate in principle that platelet function could be preserved for a much greater period of time than the current 5 days of shelf-life permitted by the FDA. This was accomplished by use of chemical compounds added as a supplement to the standard citrate anticoagulant (CPDA-1) and by modification of the geometry of the storage container. Platelet function could be demonstrated in vitro even after 15 days of storage under these conditions. The continuation of the project would have meant an examination of the practicality of this approach in terms of toxicity, efficacy, and reduction to practice in blood banking. Termination of the contract prevented initiation of the latter phases of the project.

The results presented below include our efforts to discern the role of Complement and plasmin in the platelet storage lesion. Basically, we found no direct evidence that Complement or plasmin directly damaged platelets during the storage period. However, an activation peptide from C3 conversion was present in PC in concentrations well above those shown by Polley and Nachman (J.Exp.Med. 158:603-615, 1983) to be sufficient to cause platelet activation. Also below is presented data on preservation of platelet function with various combinations of compounds added to the citrate anticoagulant. Use of inhibitors in artificial media was also explored. We have demonstrated the beneficial effects of a combination of cyclic AMP-active agents and protease inhibitors while testing many varied alternatives. Data was also gathered on the extra benefit obtained by adjusting the surface-to-volume ratio of PC in the storage bag in the presence of the best combination of inhibitors. The methods used to obtain these results are included in the description of each experimental design.

RESULTS AND DISCUSSION

The experiments performed in this project were conducted on PC either obtained directly from the local subcenter of the American Red Cross or prepared in this laboratory from whole units and half-units of blood collected from normal human volunteers. All phlebotomy procedures were reviewed by the appropriate institutional reveiw board and carried out in the presence of at least one registered Red Cross nurse. The PC were stored in PL-732 plastic containers at 22°C with continuous agitation unless otherwise noted. Samples were drawn aseptically and, when necessary, centrifuged at 14,000 x g for 2 minutes to prepare a cell-free supernate and a platelet pellet. The tests and assays employed in our studies were performed by well-published techniques or kits, or, like the analysis of GPIb by flow cytometry, are described in some detail in the appropriate subsection. The chemicals used were all reagent grade or better.

Complement

In continuing the investigation of the prior project (DAMD 17-84-C-4005) on the potential effect of plasma enzyme systems on stored platelets, we assayed for evidence of Complement activation and generation of plasmin activity during 7-10 days storage of PC. For the Complement system, we performed standard radio-immunoassay (RIA) studies to determine the amount of binding of opsonic fragments of Complement factor C3b to stored platelets. Three monoclonal antibodies were employed, directed against separate epitopes called C3c, C3g, and C3d, respectively. Table 1 shows that only the anti-C3d antibody bound significantly to stored platelets versus fresh platelets, indicating that no opsonic fragments of C3 were deposited on the platelet surface during storage. These findings, combined with our previous demonstration (Contract DAMD 17-84-C-4005) of no significant levels of the Complement membrane attack complex on stored platelets, suggest that Complement does not directly damage platelets in PC under standard storage conditions. However, limited activation of Complement, probably by the alternative pathway, was found as described below.

TABLE 1: Quantitation of Surface-Bound C3 on Fresh and Stored Platelets (mean ± SD, N=6)

Antibody	Platelets	
	Fresh	Stored
αC3c	0	4 ± 8
αC3g	208 ± 151	142 ± 132
αC3d	235 ± 218	352 ± 252 *

Data expressed as number of antibody molecules specifically bound per platelet.

*Significant difference between fresh and stored; paired t-test, p=0.04.

Evidence of the conversion of C3 to C3b and C3a was demonstrated in the supernatant plasma of standard PC by use of an RIA kit from Upjohn Diagnostics, Kalamazoo, MI. Table 2 shows a nearly linear increase in C3a over time in PC and in stored platelet-poor plasma (PPP). The equivalent increase in C3a in PPP indicates that platelets did not play a significant role in the conversion of C3 in PC. The mechanism responsible was probably that of the autocatalytic reaction involving spontaneous hydrolysis of a thiol-ester bond in C3 (Pangburn, chapter in Immunobiology of the Complement System; Ross [ed], Academic Press, 1986). The activation peptide, C3a, has been shown (Polley and Nachman, above) to cause activation and release of serotonin in washed platelet suspensions at levels as low as 10^{-11}M alone, or at 10^{-12}M when in combination with other agonists. The levels observed in PC are three orders of magnitude above the concentrations used to activate platelets, and therefore C3a may be an important agonist among those responsible for activation of platelets during storage. The lowering of Ca^{2+} by the citrate anticoagulant may partially mitigate this effect, but these results again emphasize the consideration of platelet activation as a major determinant of the status of platelets during storage.

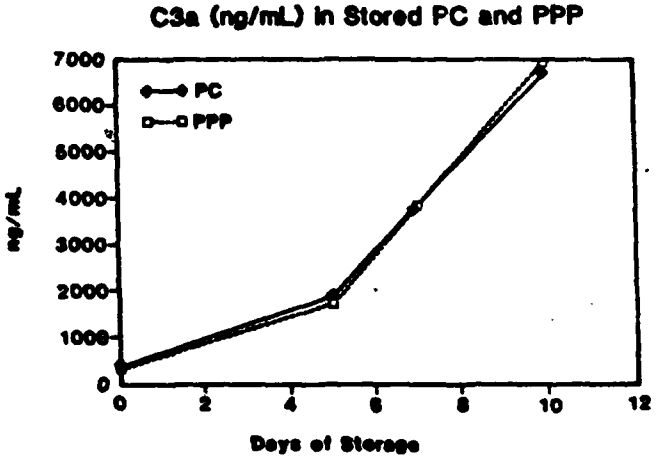

Figure 1: Mean concentration of C3a or C3a des-Arg (ng/ml) in cell-free samples from six stored platelet concentrates (PC) and the corresponding platelet-poor-plasma (PPP).

Similar assays for C5a as a result of C5 conversion showed no significant findings, all < 10 ng/mL. However, there was a 7.5% decrease noted in native C5 during 10 days of storage of PC ($p=0.04$). Whether or not this figure represents consumption of C5 in an activation pathway is unknown. An examination of stored platelets by flow cytometry for evidence of the membrane attack complex did not reveal any postive results (data collected under previous contract No. DAMD 17-84-C-4005). It would appear that the Complement system is not responsible for direct damage of platelets during the storage period.

Plasmin

An investigation of fibrinolytic activity in PC was undertaken with the premise that demonstration of plasmin generation could be linked to well-known changes in platelets during storage such as: refractoriness to thrombin stimulation, decrease in buoyant density, poor recoveries and survivals post-transfusion, and loss of glycoprotein Ib (GPIb). We assayed for plasmin activity directly in PC samples with the chromogenic substrate S-2251 and quantified only the absorbance change that was blocked by aprotinin, an inhibitor of plasmin. The results showed (Figure 2) an appreciable amount of plasmin activity in fresh and stored PC at levels significantly above ($p<0.01$) background observed in fresh or stored PPP. In addition, fibrin(ogen) degradation products (FDP) increased to a peak at 10 days, indicating breakdown in situ of fibrinogen or fibrin by plasmin in the PC (Figure 3).

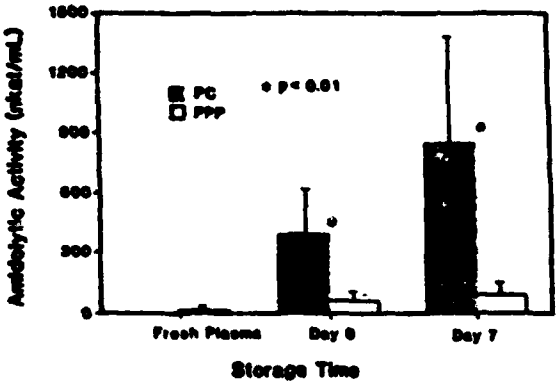

Figure 2. Plasmin-like, amidolytic activity in fresh plasma (n=6), stored PC (n=9), and stored PPP (n=5) in units of nanokatals per liter (mean ± SD).

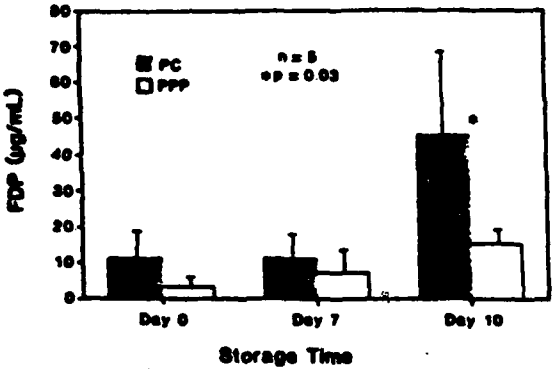

Figure 3. Fibrin(ogen) degradation products (FDP) in five PC and paired PPP in units of micrograms per milliliter (mean ± SD).

Another indication of plasmin activity in PC was noted from assays of antiplasmin activity using a chromogenic kit commercially available from Helena Labs (Beaumont, TX). There was a significant ($p=0.004$) decrease in antiplasmin levels in PC stored 7 days, but not in PPP (see Figure 4). This loss of antiplasmin activity was interpreted as evidence of generation of plasmin which complexed with the naturally occuring antiplasmin in the plasma. The total amount of plasmin generated during 7 days of stored of PC was found to be less than 3% of the amount of plasminogen in the plasma, by assay of plasminogen levels with another chromogenic kit from Helena Labs.

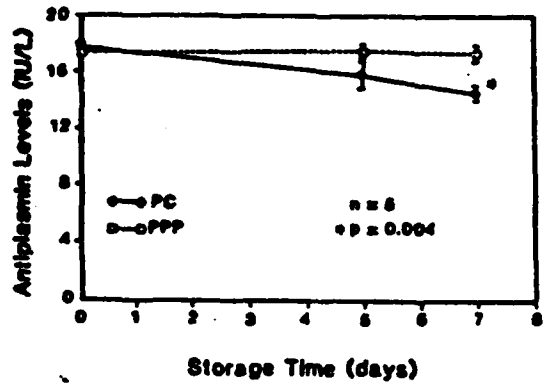

Figure 4. Antiplasmin activity levels in 5 PC and paired PPP in international units of activity per liter (mean ± SD).

GPIb

The data given above demonstrate evidence of a small amount of plasmin activity in PC. One possible consequence of this finding is an explanation for the appearance of a GPIb-negative platelet population recently described by Michelson and co-workers (J. Clin. Invest. 81:1734-1740, 1988) and by our own results with flow cytometry of stored platelets using the AN-51 monoclonal antibody. We examined non-fixed, washed platelet samples from stored PC with an indirect fluorescence technique on an EPICS-C (Coulter, Hialeah, FL) flow cytometer and compared the results to fresh platelets from the same donor on each day of analysis. An isotypic control antibody was used for non-specific binding. We found a highly significant increase in a GPIb-negative population of platelets in PC stored up to 10 days. In Figure 5 is shown an example of the changes in fluorescence patterns obtained with PC obtained from the local Red Cross subcenter. In Table 2 is given the mean values (n=8) for an integration analysis of the appearance of a GPIB-negative platelet population as defined by placement of a cursor on the fluorescence histogram. This cursor was placed at a point which bisected the overlap between the GPIb-positive platelets in fresh platelet-rich plasma and the background, non-specific staining.

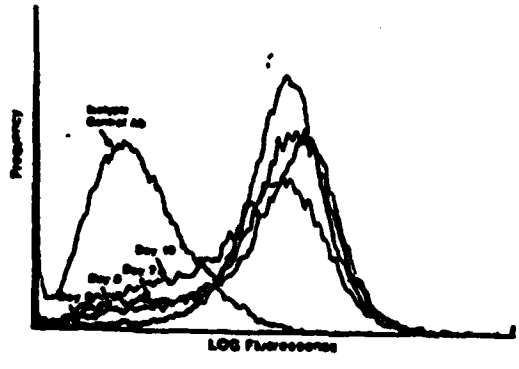

Figure 5. Overlays of flow cytometry fluorescence histograms for a typical citrated PC marked with the AN-51 antibody on Days 0,5,7, and 10.

Table 2. GPIb-negative population in stored PC (n=8).

Storage Time	% GPIb-neg.
PRP fresh	3% (overlap point)
PC Day 0	5%
PC Day 5	11%
PC Day 7	17%
PC Day 10	25%

Each time point showed a consecutive increase in the GPIB-negative population ($p \leq 0.02$).

Another expression of the data from analysis of GPIb by flow cytometry involved the use of a method described by Adelman et al. (Blood 66:423-427, 1985) to convert the mean peak channel number of the log fluorescence histogram to a linear measure of the amount of GPIb present in the overall population of platelets in one sample versus another. In this way, we compared stored platelets to fresh samples from the same (or other) normal donors to calculate a value for the percent of GPIb remaining in PC. This value fell over a ten day storage period in standard PC and correlated well with the fall in pH (r_s = - 0.77) and the rise in cell lysis indicated by plasma LDH levels (r=0.70), as shown in Figure 6.

The loss of GPIb in 10 day-old PC was also assessed by aggregation response to ristocetin (1.5 mg/mL final conc.). The calculated value of percent GPIb remaining was significantly correlated with the aggregation slope (r = 0.57) and extant of aggregation (r = 0.68), and also with the rate of recovery in the hypotonic shock test (r = 0.56) as illustrated in Table 3. These results support the notion that the fluorescence assay with the AN-51 antibody quantifies a functional part of the GPIb molecule and that the long term changes in GPIb indicate a change in functionality of stored platelets.

Table 3. Correlation of percent GPIb remaining with aggregation response to ristocetin and recovery from hypotonic shock in PC stored 10 days.

PC#	%GPIb[1]	Aggr. Slope	Aggr. ΔT	Hypo. Shock[2]
1	82%	18	50%	58%
2	81%	33	80%	25%
3	71%	13	33%	0
4	70%	14	26%	56%
5	64%	8	41%	33%
6	58%	14	32%	0
7	57%	23	48%	23%
8	57%	14	41%	43%
9	50%	21	45%	0
10	46%	9	20%	26%
11	42%	22	45%	7%
12	37%	0	0	0
13	31%	11	29%	0
14	31%	0	0	18%
$\bar{X}$ ± 1SD	56% ± 17	14 ± 9	35% ± 21	21% ± 21
PRP[3]	*	37 ± 7	98% ± 4	--

[1]Percent GPIb remaining relative to fresh PRP from a normal donor.
[2]Hypotonic shock response expressed as a percentage of the response in each PC at Day 0.
[3]Fresh PRP used as a GPIb standard and a positive control for aggregation testing (n=4).
*Arbitrary standard for 100% GPIb.

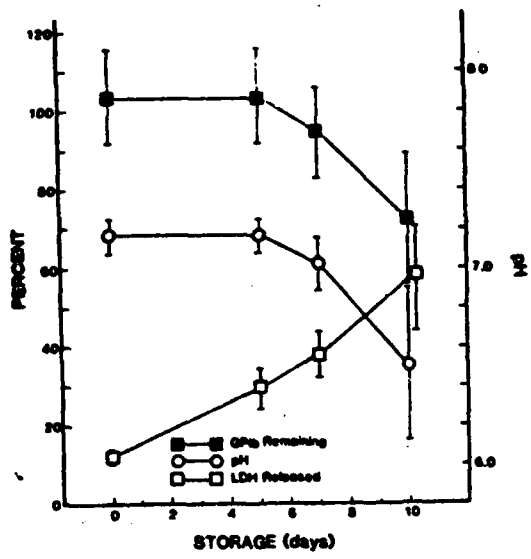

Figure 6. Correlation of % GPIb remaining in PC with plasma pH and plasma LDH levels (n=8 ± SD).

During this study of GPIb in stored PC, we attempted to block the changes over a 10 day storage period by adding protease or other inhibitors to the citrate anticoagulant before blood was collected for making PC. Two half-units of blood were collected from each of four donors per study group. One of each pair of half-units was collected into the standard citrate anticoagulant (CPDA-1); the other was collected in citrate containing an inhibitor. The PC from these half-units were stored in PL-732 containers rolled up to maintain the standard surface-to-volume ratio.

We chose to test the effects of E-amino caproic acid (EACA), a plasmin inhibitor; aprotinin, a plasmin and kallikrein inhibitor; Thromstop, a synthetic inhibitor of thrombin; leupeptin, a general protease inhibitor N-ethyl maleimide, an inhibitor of sulfhydryl-directed enzymes; and a combination of inhibitors of platelet activation, PGE-1 plus theophylline. As shown in Table 4 and Figures 7 and 8, only the inhibitors of platelet activation were successful in preventing the loss of GPIb.

Table 4. Percent GPIb remaining in citrated PC prepared in the presence of various inhibitors ("Experimental") and stored 10 days with paired, untreated PC ("Control") from the same donors (means, n=4 each group, ± 1 SD)

	Experimental	Control	Significance
1 mM EACA	65% ± 17	68% ± 24	NS, p = 0.7
348 KIU/mL Aprotinin	73% ± 13	80% ± 10	NS, p = 0.4
6 μM Thromstop	65% ± 6	69% ± 14	NS, p = 0.6
0.5 mM Leupeptin	55% ± 8	52% ± 7	NS, p = 0.4
64 μM NEM	84% ± 2	86% ± 3	NS, p = 0.4
300 nM PGE-1 plus 1.9 mM theophylline	93% ± 5	65% ± 16	*p = 0.04
PGE-1 + theophylline + Thromstop and Aprotinin	89% ± 3	53% ± 7	*p = 0.01

A formulation with Thromstop and aprotinin added to the PGE-1 and theophylline also showed remarkable preservation of GPIb, but did not significantly improve the results over the effect of PGE-1 and theophylline alone. Plasmin or other plasma protease activity apparently had no significant role in the loss of GPIb under these conditions.

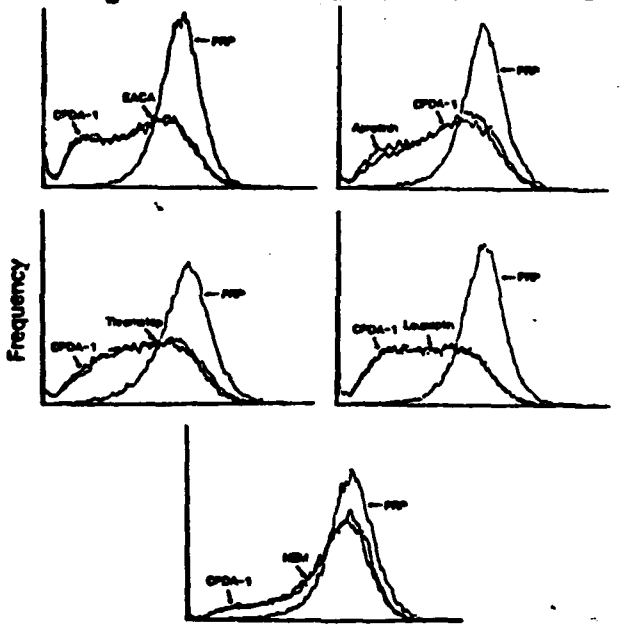

Figure 7. Overlays of fluorescence histograms for typical experiments single PC with and without inhibitor added to the anticoagulant; each pair of control and experimental PC was tested on Day 10 with the AN-51 antibody for GPIB and compared to each other as well as to fresh PRP from the same donor.

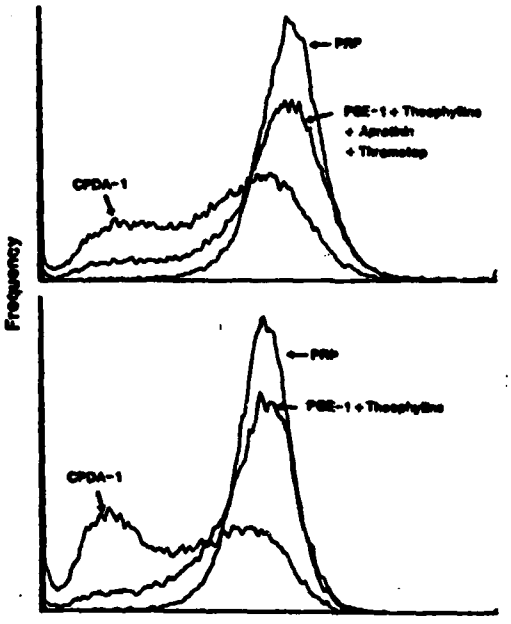

Figure 8. Overlays of fluorescence histograms for a from typical experiment from a protease single PC with and without the platelet activation inhibitors, PGE-1 and theophylline (also with Thromstop and aprotinin in upper panel), added to the anticoagulant; analyzed with AN-51 antibody for GPIB as in Fig. 7.

These results and those of the previous sections collectively demonstrate the importance of platelet activation, rather than proteolytic damage from plasma enzymes, in changes associated with the storage of platelets in PC. The working hypothesis, therefore, for improving the quality or via-bility of stored platelets was to prevent or limit the effects of platelet activation. We had demonstrated in the previous contract period (DAMD 17-84-C-4005) that the addition of PGE-1 plus theophylline and hirudin (a thrombin inhibitor) greatly extended the preservation of in vitro platelet function. The next sections of results describe further findings with various formulae and other considerations of benefit in long term storage of PC.

Inhibitors

We have examined numerous chemical and other types of modifications of PC processing and storage conditions with the goal of improving the reten-

tion of platelet function over a 10-15 day storage period as judged by in vitro markers such as hypotonic shock response, aggregation response, evidence of continued metabolic respiration (pO_2, pCO_2), rate of metabolic activity (lactate production, glucose consumption), and integrity of platelets (plasma pH, released LDH, platelet morphology). A listing of the individual agents and methods investigated in this study, without mentioning all the combinations tried, is given in Table 5.

Table 5. Anticoagulants and Additives Tested

*PGE-1 plus Theophylline	5'-AmidinoIndole
Forskolin	FUT 175
Ethanol	Calla bulb extract
Allicin (garlic)	EACA
Vitamin E	*Aprotinin
Hydrocortisone	Leupeptin
Heparin (unfractionated)	Pepstatin A
Fragmin	4°C (refrig.)
*Thromstop	Reduced Citrate
*Hirudin	High [Ca^{2+}]
PPACK	Factor-Deficient Plasma
DABE	*Reduced surface-to-volume ratio
BABIM	Elimination of second spin

The first step employed in evaluating these inhibitors was to check the reversibility of any and all effects of the compound(s) on platelets by incubating them overnite in PRP at varying doses, then resuspending the platelets the next day in autologous plasma without inhibitors and conducting the same series of function and integrity tests that was used to evaluate the platelets before addition of inhibitors. In this way, we noted no direct effects on platelet function with agents such as leupeptin, FUT-175, Thromstop, EACA, aprotinin, DABE, or allicin. Reversible effects were observed with forskolin, extract of calla lily bulbs, and with the formula of PGE-1 plus Theophylline which is illustrated by example in Table 6.

Table 6. Reversibility of PGE-1 plus Theophylline

DOSE*	PLT. COUNT**		HYPOTONIC SHOCK		AGGREGATION	
	Pre.	Post**	Pre	Post	Pre	Post
100%	96%	80%	63%	73%	0	61%
80%	95%	83%	65%	73%	0	66%
60%	95%	79%	66%	73%	0	66%
40%	96%	81%	68%	75%	0	67%
20%	95%	80%	68%	71%	0	65%
0(control)	94%	70%	84%	68%	64%	54%

*DOSE given as a percentage of the concentrations used in our platelet storage experiments (100% = 300 nM PGE-1 and 1.9 mM theophylline in PRP).

Platelet Count, Hypotonic Shock Recovery Rate, and Aggregation Vmax (to 2 x 10^{-5} M ADP) expressed as a percentage of the values in fresh PRP. Data presented here are means of 4 experiments.

**"Pre"=before washing to remove the inhibitor(s), "Post"=after washing.

Analysis of variance of the data above revealed that, before washing, there was a profound effect of PGE-1 and theophylline on platelet aggregation (as would be expected from the literature) and a subtle inhibition of hypotonic shock response ($p=0.007$). Resuspension of platelets in fresh autologous plasma resulted in complete restoration of responsiveness relative to controls. These data demonstrate that the use of PGE-1 and theophylline in novel anticoagulants for platelet storage would not result in irreversible inhibition of platelet function. Some agents had irreversible effects on platelets in this experimental design. Data from the addition of fractionated heparin, called Fragmin (Kabi Vitrum AB, Stockholm, Sweden), to PRP demonstrated an irreversible effect of platelet clumping during storage (see Table 7).

Table 7. Clumping of platelets by Fragmin.

DOSE	PLT. COUNT		HYPOTONIC SHOCK		AGGREGATION	
	Pre	Post	Pre	Post	Pre	Post
10 U/mL	64%	55%	83%	76%	73%	69%
8 U/mL	66%	70%	87%	83%	86%	81%
6 U/mL	64%	19%	84%	62%	86%	43%
4 U/mL	73%	38%	79%	77%	83%	70%
2 U/mL	76%	59%	88%	89%	92%	76%
0 (control)	91%	73%	84%	91%	65%	79%

Footnotes as in Table 6, except the Dose here is actual concentration of Fragmin in PRP.

The next step in this study was to use formulations of inhibitors as supplements to the standard CPDA-1 anticoagulant for long term storage of PC. The methodology of sterile introduction of reagents into CPDA-1 was carried out as in the previous project (DAMD 17-84-C-4005). PC were stored under standard conditions, except that the surface-to-volume ratio of the storage bag was adjusted to 4 cm^2/mL for reasons given in a following section. Many combinations of inhibitors were tried; see Table 8 for a list of 15 formulations yielding useful information. Those with unfractionated heparin or Fragmin (Formula Nos. 7 and 9) suffered from a severe clumping of platelets during a 10-15 day storage period (see Table 9). Replacement or supplementation of CPDA-1 solely with the thrombin inhibitors hirudin (Formula Nos. 3,5) or Thromstop (Formula No. 4) did not improve platelet function over that of controls over a 10 day storage period (data not shown). The previous (Contract No. DAMD 17-84-C-4005) best formulation (No. 10) containing a thrombin inhibitor plus PGE-1 and theophylline was improved by the addition of aprotinin (see Table 10).

Table 8. A partial list of formulations of anticoagulants used in studies of extended storage of PC.

(1) CPDA-1 in the standard 1:8 ratio with whole blood.

(2) CPDA-1 in a 1:12 ratio with whole blood.

(3) CPDA-1 at 1:12 with 2 antithrombin units/mL hirudin in whole blood.

(4) CPDA-1 at 1:8 with 6 μM Thromstop in whole blood.

(5) No CPDA-1; 8 units/mL hirudin dissolved at 8X in 0.9% NaCl for a 1:8 ratio with whole blood, with dextrose and phosphate added at the levels in CPDA-1.

(6) No CPDA-1; 8 units/mL hirudin plus 300 nM PGE-1 and 1.9 mM theophylline with 1 x 10^{-4}M 5-Amidinoindole dissolved at 8X in 0.9% NaCl for a 1:8 ratio with whole blood with dextrose and phosphate added at the levels in CPDA-1.

(7) No CPDA-1; 10 u/mL heparin plus 300 nM PGE-1 and 1.9 mM theophylline dissolved at 8X in 0.9% NaCl for a 1:8 ratio with whole blood, with dextrose and phosphate added at the levels in CPDA-1.

(8) CPDA-1 at 1:8 with 300 nM PGE-1 and 1.9 mM theophylline in whole blood.

(9) Formula (8) with 20 anti-Xa units/mL Fragmin in whole blood.

(10) Formula (8) with 8 units/mL hirudin in whole blood.

(11) Formula (8) plus 348 kallikrein inhibitor units/mL aprotinin in whole blood.

(12) Formula (11) plus 6 μM Thromstop in whole blood.

(13) Formula (11) plus 8 u/mL hirudin in whole blood.

(14) Formula (11) plus 2 x 10^{-5} M DABE in whole blood.

(15) Formula (10) plus 40 μM BABIM.

Table 9. Use of heparin in extended storage of PC.

	% plts[1]	pH	pO_2	pCO_2	Shock[2]	LDH[3]	%Discs[4]
Day 10							
Heparin (For. 7)[5]	63%	7.31	174	14	98%	35%	24%
Citrate + Fragmin (For. 9)	58%	6.88	150	11	92%	ND	23%
Citrate (For. 8)	75%	6.76	109	16	74%	19%	48%
Day 15							
Heparin (For. 7)	33%	6.82	186*	8*	38%	43%	16%
Citrate + Fragmin (For. 9)	39%	6.35	185*	5*	0	ND	4%
Citrate (For. 8)	61%	6.28	117	10	17%	23%	20%

[1]Countable platelets remaining relative to the fresh PC at Day 0.

[2]Hypotonic shock response expressed as a percent of the response in the fresh PC at Day 0.

[3]Plasma levels of LDH expressed as a percent of the total releaseable LDH in PC.

[4]Percentage of platelets remaining in the discoid shape.

[5]Formula number from Table 8.

*These values of pO_2, pCO_2 represent approximate equilibration with room air, suggesting no current respiratory activity in the PC.

Table 10. Storage of PC for 15 days in CPDA-1 containing PGE-1 and theophylline with or without aprotinin and/or a thrombin inhibitor; means only, n ≥ 4 in each group.

Additive(s)[1]	Formula*	%Plts	pH	pO_2	pCO_2	Shock	ADP[2]	LDH	%Discs
Hirudin	No. 10	ND	6.48	136	14	36%	11%	31%	37%
Aprotinin	No. 11	69%	6.41	116	22	65%	0	20%	52%
Aprotinin + 6 μM Thromstop	No. 12	70%	6.55	103	25	66%	76%	11%	68%
Aprotinin + 1 μM Thromstop	No. 12(mod)	77%	6.27	126	20	59%	26%	8%	ND
Aprotinin + Hirudin	No. 13	67%	6.46	85	29	87%	42%	11%	70%

*Formula number from Table 8.

[1]Additives to the basic anticoagulant formulation of CPDA-1 plus PGE-1 and theophylline (Formula No. 8).

[2]Aggregation response induced with 2 x 10^{-5}M ADP in terms of a percentage of the aggregation slope produced by fresh platelets.

Although the need for inhibitors of platelet activation and thrombin has been discussed, the basis of the additional benefit with aprotinin is not clear. Besides its action as an inhibitors of plasmin and kallikrein proteolytic activity, aprotinin has been reported to reduce the appearance of platelet secretion products in whole blood (Lundsgaard-Hansen, Vox Sang. 45:1-5, 1983). Again, it would appear that efforts to reduce the activation (and secretion) reactions of platelets help preserve platelet function during storage. Electron micrographs of platelets stored in the presence of citrate plus PGE-1, theophylline, Thromstop, and aprotinin (Formula No. 12) reveal generally intact morphology and internal structures in the majority of platelets even after 15 days of storage (see Figure 9). Other agents, such as the Factor Xa inhibitors DABE and BABIM (Formula Nos. 14 and 15), did not provide additional improvement (data not shown).

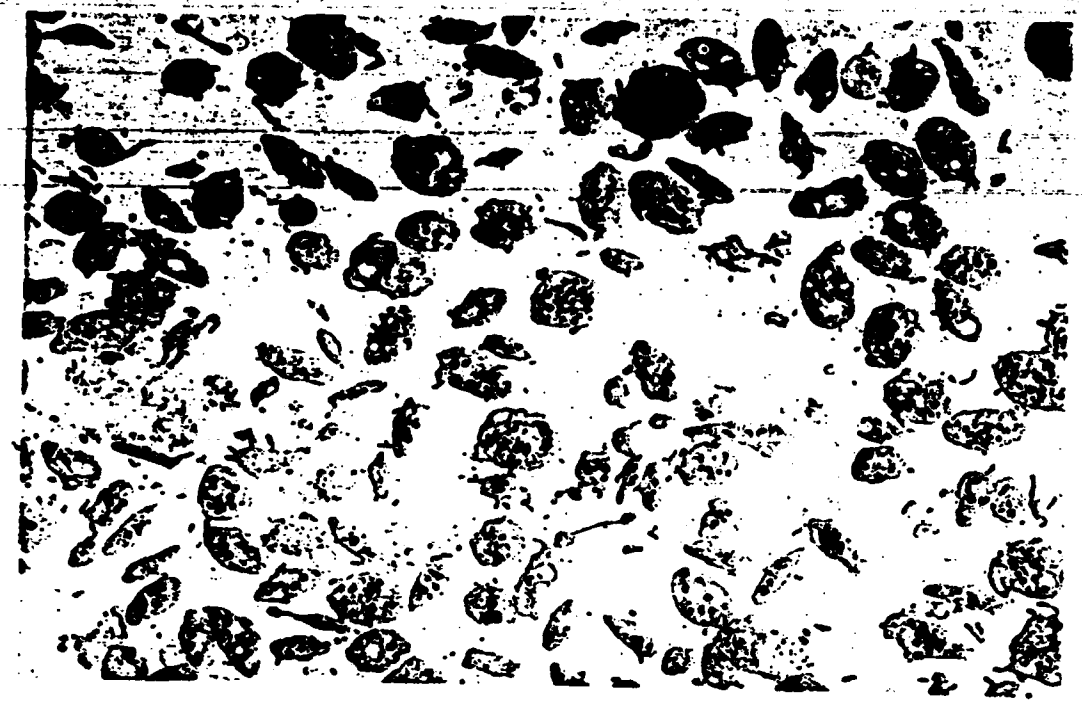

Figure 9a. Electron micrograph of PC in CPDA-1 plus PGE-1, theophylline, Thromstop, and aprotinin at Day 15 of storage (x4,450).

Figure 9b. Electron micrograph of same PC at Day 15 (x21,800).

Artificial Media

The success of platelet activation inhibitors in plasma PC prompted a similar approach to the improvement of platelet storage in artificial media. Our collaborators, Andrew Heaton, MD, and Stein Holme, PhD, of the American National Red Cross Blood Services at the Tidewater Region headquarters in Norfolk, VA, have devised a nutritive electrolyte solution for platelets which appears to improve the ability of cells to withstand extended storage periods (see Holme et al., Br. J. Haematol. 66:233-238, 1987). We found that with the addition of platelet activation inhibitors, the storage period in artificial media could be extended to as long as 20 days before a substantial loss of *in vitro* platelet function was evident. This was accomplished without direct modification of the anticoagulant. Units of non-aspirinated CPDA-1 whole blood were selected at random from Red Cross bloodmobiles and centrifuged to make platelet-rich plasma in our laboratory. To the PRP was added one eighth volume of citrated saline containing the supplement of inhibitors, such as PGE-1 and theophylline, dissolved at high concentrations in the citrated saline to give effective levels in the PRP equivalent to those obtained in blood from modified CPDA-1. After a short incubation (15-30 minutes) the PRP was centrifuged to make a platelet pellet which was resuspended in the Norfolk artificial medium containing another dose of the inhibitors. This approach seemed to be more effective than the use of inhibitors in whole blood, possibly due to metabolizing of PGE-1 by RBCs and thus a decrease in the effect of this inhibitor on platelets.

Data obtained through the addition of PGE-1 plus theophylline to PRP and the Norfolk medium are presented in Table 11. A further improvement was noted with the addition of aprotinin and Thromstop to the PGE-1 plus theophylline cocktail (see Table 12). These results have been confirmed by Drs. Heaton and Holme in their laboratory with markers of platelet integrity and metabolic status. Just prior to termination of the contract, we also tested a modification of the Norfolk medium to reduce the amount of phosphate ion; the CPD reagent in the medium was replaced with ACD (PO_4-free) and 10% more sodium bicarbonate was added to equilibrate the pH. The phosphate ion concentration was reduced to examine its effect on platelet metabolism during storage. The results (Table 13) suggest that the reduction in phosphate was beneficial in lowering the metabolic rate of stored platelets without compromising function and integrity.

Table 11. PC stored in PAS + glucose (the Norfolk medium) with 300 nM PGE-1 plus 1.9 mM theophylline (n=10).

	Count[1]	pH	pO_2	pCO_2	Shock[2]	Glucose	Lactate	%LDH[3]
Day 1	1.20	7.18	34	89	0.090	532 mg/dL	2.6 mM	2%
Day 15	80%	7.04	82	33	79%	188 mg/dL	31 mM	12%
Day 20	69%	6.72	120	21	58%	101 mg/dL	40 mM	17%

[1]Platelet count expressed per 10^9/mL; Day 15 and Day 20 value expressed as a percent of Day 1.
[2]Hypotonic shock recovery rate expressed as Δ OD/min; Day 15 and Day 20 value expressed as a percent of Day 1.
[3]Released LDH expressed as a percent of total cellular LDH.

Table 12. PC stored in PAS + glucose (the Norfolk medium) with 300 nM PGE-1 + 1.9 mM theophylline + 348 KI u/mL aprotinin + 6 μM Thromstop (n = 6).

	Count	pH	pO_2	pCO_2	Shock	Glucose	Lactate	%LDH
Day 1	1.20	7.09	15	99	0.091	522 mg/dL	3 mM	2%
Day 15	97%	6.78	75	37	78%	179 mg/dL	28 mM	6%
Day 20	90%	6.56	130	24	62%	92 mg/dL	36 mM	10%

Footnotes as in Table 11.

Table 13. PC stored in PAS + glucose + 300 nM PGE-1 and 1.9 mM theophylline with ACD substituted for CPD in PAS (n=3).

	Count	pH	pO_2	pCO_2	Shock	Glucose	Lactate	%LDH
Day 1	1.40	6.92	32	127	0.122	556 mg/dL	4.6 mM	1%
Day 15	96%	6.72	53	37	82%	196 mg/dL	31 mM	6%
Day 20	93%	6.66	97	29	73%	123 mg/dL	33 mM	8%

Footnotes as in Table 11.

Other formulations of inhibitors were tried in the use of the Norfolk artificial medium for extended storage of platelets; none proved as effective as those described in Tables 11-13. These attempts included preparations with 100 μM caffeine with and without PGE-1, 300 nM PGE-1 without theophylline, 300 nM PGE-1 with 1 mM theophylline (half-strength), 10 μM trifenagrel (an inhibitor of the cyclo-oxygenase enzyme system in platelets), forskolin at several concentrations, and changes in methodology: such as, adding PGE-1 plus theophylline to the PRP only or to the PC only instead of at both steps. The lack of success with these maneuvers illustrates the extent of the role of platelet activation in the storage lesion. While much more investigation would be needed to identify and address the problem areas, we feel that the combined approach of platelet activation inhibitors and artificial media could lead to a practical solution for extending the useful shelf-life of PC to 15-20 days.

Surface-to-Volume Ratios

In our first studies on the addition of platelet activation inhibitors to the citrate anticoagulant for plasma PC, we used the standard ratio of bag surface to PC volume of 7 cm^2/mL. Upon noting a decrease in lactate build-up and glucose consumption in PC as a result of the inhibitors, we decided to see how much we could minimize the bag surface area before compromising gas exchange across the bag wall. To accomplish this strategy experimentally, we rolled up the PL-732 storage container length-wise and secured it with metal clips before introducing PC into the bag for storage. The initial experiments were done with the formulation of CPDA-1 modified with 300 nM PGE-1 plus 1.9 mM theophylline and 348 KI units/mL aprotinin (final concs. in PRP); the PC were stored in their native plasma (containing the inhibitors) at surface-to-volume (S/V) ratios of 7,4, and 2 cm^2/mL. The results (Table 14) showed a remarkable effect of S/V ratio on the outcome after 15 days of storage of these PC. The best S/V ratio appeared to be 4cm^2/mL, which was later confirmed for other formulations of inhibitors.

Table 14. Effects of surface-to-volume ratio in PC stored in CPDA-1 + PGE-1 + theophylline + aprotinin for 15 days. (Means, N=4 each group).

S/V	Percent Plts Remaining	pH	pCO_2	pO_2	% Hypo Shock Remaining
2	ND	5.84	7	187	0%
4	61%	6.58	18	135	81%
7	31%	6.61*	9	180	19%

* PC having $pCO_2 \leq 10$ and $pO_2 \geq 180$ mm Hg and minimal or no response to hypotonic shock are probably not of value for transfusion even if the pH is still above 6.0.

The beneficial effect of a reduced S/V ratio was used to advantage in all of the successful formulations of inhibitors for PC stored in plasma and for PC stored in artificial media. The data reported in Tables 10-14 of this report were generated with PC stored at S/V = 4. When control studies were performed at reduced S/V ratios without platelet activation inhibitors, the results indicated no beneficial effect due solely to a reduction in surface area (data not shown). It would appear also, mostly from the data in Table 14 and other similar experiments, that the full beneficial effect of the platelet activation inhibitors can not be realized until the S/V ratio is lowered to take advantage of the lower metabolic rate and reduced need for gas exchange across the storage container. Possibly, this effect is mediated by a reduction in the rate of exposure of stored platelets to a foreign surface, namely the bag wall, by reducing the available surface area with which the platelets can interact. The ability of the inhibitors to prevent activation by this route may be greatly enhanced when the number of collisions of platelets with the bag wall is statistically reduced by this method.

Future Directions

This report presents data supporting the notion that platelet activation is largely responsible for the loss of platelet function and integrity in PC. Our use of inhibitors of platelet activation and reduced surface-to-volume ratios has shown significant improvements in preserving platelets *in vitro* for periods up to 15-20 days. The second part of this contract would have examined the issues concerning application of this technology to transfusion practice. Platelet survival and efficacy studies need to be done in animal models and in man to examine the *in vivo* circulation of platelets stored in the presence of activation inhibitors. Toxicity also should be investigated, although most of the compounds in the successful formulations have been used in man at levels many times higher than that employed here without acute toxic effects. Only after these studies are completed can we judge the practicality of the use of our approach in improving the shelf-life of stored platelet concentrates.

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